BIO-DEGRADATION OF CYANIDE OF COKE COAL UNIT OF ROURKELA STEEL PLANT

A PROJECT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

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By

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CERTIFICATE

This is to certify that the research project report entitled **"Bio-Degradation of Cyanide of Coke Coal Unit Of Rourkela Steel Plant"** submitted by **Miss Preety S. Gedam** in partial fulfillment of the requirements for the award of the degree of Master of Technology in Biotechnology and Medical engineering with specialization in Biotechnology at the National Institute of Technology, Rourkela is an authentic work carried out by her under my supervision, co-supervision and guidance.

To the best of my knowledge, the matter embodied in the report has not been submitted to anyother University/Institute for the award of any Degree or Diploma.

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LIST OF ABBREVIATION

Serial No.	LIST OF ABBREVIATION	FULL FORM
1	temp	Temperature
2	HCl	Hydrochloric acid
3	H_2O_2	Hydrogen peroxide
4	µg	Microgram
5	µl	Microlitre
6	m	Meter
7	mM	Millimolar
8	mL	Millilitre
10	M	Molar
11	cm	Centimeter
12	gm	Gram
13	Hrs	Hours
14	NA	Nutrient Agar
15	RSP	Rourkela Steel Plant
16	GAC	Granular Activated Carbon
17	H_2SO_4	Sulphuric acid
18	*et al*	And others

ABSTRACT

Cyanide is a toxic compound which is produced by some foods, certain microorganisms, plants as well as some animals. Though their production by the above sources is less, metal finishing as well as mining industries are the main cause of cyanide pollution. However cyanide is used for extraction of gold from ores, electroplating, coal gasification etc; their occurrence in large scale in the environment is highly hazardous for the living beings. To avoid these hazards Central Pollution Control Board (CPCB) has set minimal cyanide level in effluent as 0.2 mg/L. In order to achieve this, various physical, chemical as well as biological methods were adopted. Biological methods were found to be most useful and less expensive compared to others; which include use of algae, fungi and bacterial cultures. In the present study, bacterial colonies were isolated from the water sample of AT I and efficient cyanide degrading colony was isolated. Various process parameters such as pH, temperature, and inoculum size were studied and optimized for obtaining maximum cyanide degrading efficiency. A comparative study between Granular Activated Carbon (GAC) and clinker was carried out in order to identify their adsorption capacities. Optimum conditions for biodegradation were obtained and a simultaneous adsorption and biodegradation (SAB) study was carried out at neutral pH of 6.5-7.5, temperature 30°C, and inoculum size of 1 mL. Using SAB process, maximum cyanide degradation was found to be 99.16 %.

Keywords: Cyanide, RSP (Rourkela Steel Plant), Bacteria, GAC (Granular Activated Carbon), SAB (simultaneous adsorption and biodegradation)

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Cyanide is a chemical compound that contains monovalent combining group known as cyano group abbreviated as –CN, consists of a carbon atom which is triple bonded to nitrogen atom. In nature cyanide occurs in organic as well as inorganic form. Organic cyanides are usually called as nitriles such as acetonitrile. Inorganic cyanides are present as the negatively charged polyatomic cyanides such as sodium cyanide, potassium cyanide, ferro-cyanide which are highly toxic.

Cyanide is produced by some foods, such as lima beans and almonds. Certain microorganisms like bacteria, algae and fungi; as well as certain plant and animal species produce cyanide.(Dzombak, Ghosh et al. 2010)

Though their production by these sources is less, metal finishing and mining industries are the main cause of occurrence of cyanide in bulk amount in the environment, mainly where it is used for the extraction of gold from ores, electroplating, precious metal refining, aluminum electrolysis, coal gasification, coal coking, as well as by the production of pharma-ceuticals, synthetic fibers, and plastics.(Yanase, Sakamoto et al. 2000)

Cyanide is a well known metabolic inhibitor and is listed among the 65 toxic pollutants in the Effluent guideline standards given in title 40, section 400- 475, of the code of federal regulations (Code of Federal Regulations, 2008). Cyanide ion –CN and free cyanide as HCN is the primary toxic agent. It can enter the body through inhalation, ingestion or absorption through the eyes and skin. Inhalation of 20-40 parts per million of gaseous HCN may cause headache, drowsiness, vertigo, weak and rapid pulse, deep and rapid breathing, a bright-red color in the face, nausea and vomiting followed by convulsions, dilated pupils, clammy skin, a weaker and more rapid pulse and slower, shallower breathing. Inhalation of 100-300 parts per million of gaseous HCN may results in death within 10-60 minutes whereas inhalation of 2000 parts per million of gaseous HCN many results in death within 1 minute(Gibbons 2005).

Some electroplating and metal finishing plants stores cyanide wastes for years, which leads the effluent to contain 10,000‑30,000 mg/L of cyanide (Wild, Rudd et al. 1994)

In order to protect environment as well as the water bodies, cyanide-containing effluents should be subjected to treatment prior to its discharge in the environment. According to Environmental Protection Agency (EPA), the proposed level of discharge of cyanide in the drinking and aquatic water is 200 and 50 parts per billion respectively. Whereas according to Central Pollution Control Board (CPCB), cyanide level in effluent should be 0.2 mg/L. Keeping view on these considerations it is necessary to reduce the cyanide concentration below regulatory level.

Cyanide-contaminated water having free cyanide (HCN, CN^-) and cyanide that is weakly bonded to metals is often treated by alkaline chlorination or biological oxidation process(Akcil, Karahan et al. 2003); whereas various other treatment methods have been developed for cyanide that is strongly bonded or complexed with metals which cannot be treated by above methods. Most of the methods are based on cyanide recovery using acidification or destruction by chemical oxidation technique; which comes with high reagent costs. Alkaline-chlorination-oxidation process is one of the methods which can be used for simple forms of cyanide; whereas for cyanide complexed with metals this process is not effective due to slow reaction rates(Patil and Paknikar 2000).Alternative treatment methods for cyanide containing wastes include copper-catalyzed hydrogen peroxide oxidation (Kitis, Karakaya et al. 2005), ozonation (Parga, Shukla et al. 2003), electrolytic decomposition (Parga, Shukla et al. 2003), etc. Although physical and chemical methods have proven to be useful in removal of cyanide they are found to be expensive as well as complex to operate. Biological treatment methods are considered as cost effective from economic stand point(Ebbs 2004). Several plant species have been proven significant for phytoremediation of cyanide compounds(Aksu, Calik et al. 1999). Various algal species also proven to degrade cyanide at a considerable level which includes *Arthrospira maxima, Chlorella species* and *Scenedesmus obliquus*(Gurbuz, Ciftci et al. 2004). Fungal species like *Fusarium solani, Fusarium oxysporum, Trichoderma polysporum, Scytalidium thermophilum* and*Penicillin miczynski* shows iron cyanide degrading capacity even at lower pH values.

Several microbial species uses cyanide as their sole nitrogen or carbon source and effectively degrade cyanide into less toxic products. Bacteria can convert free and complexed cyanides to bicarbonates and ammonia. *Pseudomonas species* like *Pseudomonas fluorescens, Pseudomonas Acidovorans, Pseudomonasputida, Pseudomonas stutzeri, Pseudomonas pseudoalcaligenes, Pseudomonas Acidovorans,*as well as*Eschericia coli* BCN6, *Bacillus magaterium, Bacillus*

*pumilis*etc have shown remarkable degradation of cyanide. However, microbial degradation depends on the factors like pH, temperature, nutrient availability, oxygen level and inoculum size. Biodegradation is also affected by concentration of cyanide present in the water or soil sample as for some microbial species cyanide at higher concentration may be toxic. (Dash, Gaur et al. 2009)

Present study focuses on the degradation of cyanide from contaminated water of coke-coal unit of Rourkela Steel Plant (RSP). Adsorption and biodegradation methods are used separately as well as simultaneously for the study.

OBJECTIVES OF STUDY:

- Characterization of the cyanide containing waste water
- Isolation and screening of the microbial flora
- Gram staining and biochemical characterization of the isolated bacteria
- Analysis of cyanide degradation capacity of the isolated species
- Parameter optimization of the microbial species for cyanide degradation efficiency
- Analysis of cyanide removal using clinker as an adsorbent
- Comparative study between granular activated carbon and clinker for cyanide removal
- Analysis of simultaneous adsorption and biodegradation using microbial species and clinker

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Toxicity and environmental hazards of chemicals have been studied from three decades. Cyanide tastes super bitter; hence it is very difficult for a normal person to consume. Physical effects of cyanide are distinctive from other hazardous chemicals. As per American Association of Poison Centers, in 2013 approximately eight people died from cyanide poisoning.

Cyanide has a long history of poisoning, which includes Nazi leader's suicide, death of Jonestown cult followers in 1978.Cyanide found in peach pits, describes the term "death by peach" describing its hazard. Cyanide also exists in apricot, apple seeds, lima beans, cassava as well as almonds as a natural poison. As it is used in textile and plastic industries as well as paper manufacturing, it can enter through inhalation of smoke containing cyanide during fires.

Government has restricted on the selling of cyanides as cyanide, arsenic, and strychnine are considered to be the most poisonous compounds, hence making it difficult for normal person to find it. According to Deborah Blum, cyanide blocks cells from using oxygen which results in chemical strangulation by affecting certain enzymes and biological processes. Health hazards from cyanide begins at the levels of 50 parts per million whereas safe exposure should not exceed 10 parts per million.

Cyanide can be obtained in gaseous form like hydrogen cyanide or can be found in white powdered form like sodium cyanide and potassium cyanide. According to Wall Street Journal, KGB used cyanide gas sprayed from a spray gun to kill Ukrainian opposition leaders in 1950.

912 people died of a cool aid like drink having trace amounts of potassium cyanide in 1978. In 1982, eight people died in Chicago because of the consumption of Tylenol which was laced with cyanide; the poisoner was never found. In 2013, cyanide from local gold-mining industry was dumped into a watering hole in Zimbabwe; causing death of about 300 elephants, along with lots of other animals; whereas other animals which did not consume water directly died by eating poisoned animals. Cyanide can work as a poison even in tiny amounts, which is difficult to be recovered from tissues or the blood samples.

Never-solved 1982 Tylenol murders in Chicago marked the hazards of cyanide. In September 1982, Tylenol capsules were removed from the drug stores as they were laced with cyanide and killed eight people in Chicago. Tests proved that the capsules contained potassium cyanide at a level toxic enough to provide various fatal doses. Common poisoning forms of cyanide are hydrogen cyanide, sodium cyanide and potassium cyanide(Fletcher 2009).

Though metal finishing and mining industries, extraction of gold from ores, electroplating, precious metal refining, aluminum electrolysis, coal gasification, coal coking are some of main sources; cyanide can also be liberated by tobacco smoke in the environment (Osobamiro 2013).

2.1 Methods of cyanide analysis

Various methods for cyanide analysis are as follows:

2.1.1 Spectrophotometric method

Looking at the hazards of cyanide, it is very important to analyze whether cyanide is present in the respective sample or not. Various methods had been derived for this purpose which includes spectrophotometric analysis. This method is very sensitive and can be used for trace determination of cyanide with ninhydrin in an alkaline medium; ninhydrin is an inexpensive and single reagent required for the process. For the range of 0.04 – 0.24 µg cm^{-3}, absorption can be checked at 590 nm. For various concentrations of cyanide recoveries can be obtained between 96.8 – 100 %(Nagaraja, Kumar et al. 2002)

Cyanide in tobacco plant can also be determined spectrophotometrically. Pyridine-barbituric acid can be used as a colour forming reagent and concentration was measured at wavelength of 578 nm.

2.1.2 Spectrophotometric method following micro distillation

The catalytic reaction of ninhydrin in the presence of cyanide causes formation of CN-NH complex, based on the absorption of this complex, trace analysis of cyanide can be done. Micro distillation conditions can be optimized and seen that cyanide content depends on the distillation time. A wavelength of 598 nm can be used for cyanide concentration in a range of 40 – 160

µg/L. Cyanide recoveries in the range of 76.2-89.2%, 73.2-91.2% and 76.8-94.8% can be obtained. (Chueachot and Chanthai 2014)

2.1.3 Chromatographic technique

Ion exchange chromatography (IEC) with pulsed amperometric detection method (PAD) can be used for cyanide detection. Cyanide containing samples can be digested using UV or acid to convert them into hydrogen cyanide gas which can be distilled to NaOH; after which total cyanide can be determined by spectrophotometrically or by titration method. But, this process is complicated as well as it faces interferences from high pH solutions, oxidizers as well as sulfur containing compounds. IEC method can eliminate such complications whereas PAD method is selective, sensitive and suitable for direct detection of cyanide.

2.2 Methods for treatment of cyanide

Various chemical, physical as well as biological methods are described below

2.2.1 Chemical and physical methods

Various chemical and physical methods had been acquired for cyanide removal. Usage of quaternary ammonium salts is one of the methods in which cyanide is removed by precipitation as some of the minerals contain copper species which are used for metal extraction, these copper species may react with cyanide and form cuprocyanide complex; which can cause high cyanide consumption. (Alonso-González, Nava-Alonso et al. 2009)

Copper-impregnated pumices and hydrogen peroxide together also enhanced the initial rate and extent of cyanide removal. However it is affected by certain parameters like particle size fractions, pH as well as temperature. Copper-impregnated pumice is a cheap, readily available, natural, and porous heterogeneous catalyst which may be an effective treatment technology for cyanide removal from solution(Kitis, Karakaya et al. 2005)

Electrolytic decomposition is one of the methods, which depends on steam cost, electricity cost as well as water costs. The equipment which is used for electrolytic decomposition is simple to handle, it does not take much attention while operating, while destroying cyanide, and other toxic products are not formed in this process. (Easton 1967)

Adsorption is one of the widely used technologies for removal of cyanide. Plain and metal-impregnated activated carbons are proved to be efficient adsorbents. Powdered activated carbon had been examined for its feasibility to remove cyanide (Adams 1994); however high cost of adsorbents as well as capital loss for their regeneration, had marked a big disadvantage over their uses, hence biological methods have given an inexpensive and environment friendly alternative to conventional chemical and physical processes.

2.2.2 Bioremoval of cyanide

Particular attention is being focused upon the use of biological treatment as an inexpensive method either alone or along with, chemical and physical treatment processes. Aerobic and anaerobic microbial treatment processes have been successfully employed in the destruction or removal of organic compounds.

(a) Phytoremediation

Phytoremediation can be used as a promising method for cyanide removal, Hybrid willows (Salix matsudana Koidz · Salix alba L.), weeping willows (Salix babylonica L.) and hankow willows (Salix matsudana Koidz)have shown to reduce cyanide from three different sources as water, air and aerial tissues of plants. In hypotonic solution, plant shows considerable uptake of cyanide hence showing the disappearance kinetics.

Plants show a passage of ferrocyanide through it, resulting in detection of very small amounts of ferrocyanide in the sample. Due to transpiration, no cyanide can be found in the air. In hydroponic solution, large fraction of mass can be metabolized during transportation in the plants. (Yu, Zhou et al. 2006)

(b) Algal degradation

Algal cultures like *Arthrospira maxima, Chlorella species* and *Scenedesmus obliquus* shows promising results in degrading cyanide; although algal degradation is affected by parameters as pH, initial concentration of algal cells, temperature and cyanide concentration. *A. maxima*cannot survive in the higher cyanide concentrations due to its sensitivity. At pH value of 10.3 (alkaline pH), and initial cyanide concentration ranging between 50 and 100 mg/L, *Chlorella*

*species*shows removal up to 86 %; whereas *Scenedesmus obliquus*shows removal up to 99 %. (Gurbuz, Ciftci et al. 2009)

*Scenedesmus obliquus*shows reduction of effluents containing weak acid dissociable cyanidein a gold mill to 6 mg/L from 77.9 mg/L in 77 hours.*Scenedesmus obliquus* is grown in SAG media, the compositionis given below:

Ingredients	**Grams/litre**
KNO3	0.1
K2HPO4· 7H2O	0.02
MgSO4· 7H2O	0.001
FeCl3· 6H2O	0.001
Sample	50 ml

It is found that *S. obliquus*can degrade cyanide by uptake of metals like Zn, Fe and Cu.

(c) Fungal biodegradation

For alkaline solutions, of pH range 9.2 - 10.7; fungal strain *Fusarium solani* shows a good potential for degrading cyanide. Yeast extract should be added to the medium as a nutrient source. *F. solani* follows a hydrolytic detoxification pathway. It does not require prior acidification step, hence risk of cyanhydric acid volatilization step can be avoided. Many industrial alkaline effluents can be treated with the help of *F. solani*(Dumestre, Chone et al. 1997).

*Fusarium oxysporum*utilizes metal cyano compounds as their sole source of nitrogen. Intact mycelia and cell-free extract of *F. oxysporum* catalyzes the hydrolysis of tetracyanonickelate (TCN). Enzymes hydratase and amidase brings about the degradation of TCN(Yanase, Sakamoto et al. 2000).

Basidiomycetes fungal strains like *Polyporus arcularius, Schizophyllum commune* and *Ganoderma lucidum* shows more cyanide degradation activity as compared to other fungal strains like *Clava riadelphus truncates, Pleurotus eryngii, Ganoderma applanatum, Trametes*

versicolor and *Cerrena unicolor*. Certain parameters like amount of biomass, temperature, pH, agitation rate, initial cyanide concentration should be optimized for the fungal strains. (Özel, Gedikli et al. 2010)

(d) Bacterial degradation

For biological degradation, bacteria mainly follow four pathways as: hydrolytic, oxidative, reductive, and substitution/transfer. (Sirianuntapiboon, Chairattanawan et al. 2008)

Azotobactor vinelandii is a N_2fixing bacterium, containing nitrogenase enzyme which is mainly considered to be involved in cyanide degradation.*A. vinelandii* is been seen to grow and remove cyanide from cassava mill waste water. As this water posses high organics and cyanide content; it is highly toxic and can cause serious threats to environment as well as aquatic life. Degradation of cyanide by *A. vinelandii* is strongly affected by initial cyanide concentration. As cyanide concentration increases, degradation rate also increases. Cells at exponential phase degrade cyanide more rapidly compared to the stationary phase. In an activated sludge system *A. vinelandii* shows about 90 % of the cyanide removal(Kaewkannetra, Imai et al. 2009).

Pseudomonas pseudoalcaligenes shows removal of cyanide for alkaline solution in a batch reactor. pH and dissolved oxygen concentration are considered to be important parameters for *P. pseudoalcaligenes*. Alkaline pH helps to avoid volatilization of hydrogen cyanide (HCN). The combination of a high pH and low dissolved oxygen saturation reduces the release of HCN.*P. pseudoalcaligenes* can degrade cyanide at a rate about 0.64mgCN/ L^{h-1} in a medium containing acetate as a carbon source, under controlled conditions of pH and dissolved oxygen concentration (Huertas, Sáez et al. 2010).

Pseudomonas fluorescens uses ferrocyanide as a sole source of nitrogen. Degradation of cyanide by *P. fluorescens* depends on the parameters like pH, agitation and initial cyanide conditions.

The nutrient composition for *P. fluorescens* is as follows:

Ingredients	**Grams/litre**
Glucose	5

Peptone	1
Yeast extract	1
KH_2PO_4	0.5
K_2HPO_4	0.5
NH_4SO_4	0.5
$MgSO_4$	0.05

The cells were transferred to growth medium having glucose and ferrocyanide as a source of carbon and nitrogen replacing yeast extract, peptone and NH_4SO_4.

For initial cyanide concentrations of 50, 100,200 and 300 mg/L at a neutral pH, cyanide degradation efficiency by *P. fluorescens* can be achieved up to 96.4, 94.1, 86.2 and 69.3% respectively; whereas if simultaneous adsorption and biodegradation (SAB) process is used, removal efficiency of 99.9 % can be achieved for 50, 100mg/L initial cyanide concentrations.

2.3 Physical characteristics of water

Various physical properties of water are discussed below:

2.3.1 Dissolved oxygen

Number of micromoles of oxygen gas per kilogram of water is defined as dissolved oxygen concentration (Montgomery, Thom et al. 1964). Oxygen is an essential element for all life forms; dissolved oxygen in a concentration of 110 percent is necessary but if it exceeds the limit, can cause the aquatic life to suffer from gas bubble disease. In contrast, if dissolved oxygen level falls below the level, fish death rate will increase, certain fishes will not be able to reproduce as well as bacterial growth will be hampered(Helm, Jalukse et al. 2012).

2.3.2 Biochemical oxygen demand

Amount of oxygen that is consumed by living aquatic organisms by oxidizing organic matter is referred as biochemical oxygen demand.

Organic wastes like leaves, dead plants, manure, and sewage as well as food waste which are present in the water supply are decomposed by the microorganisms hence causing their

decomposition. During the process, aerobic microbes consumes dissolved oxygen present in the water, BOD is the measure of the oxygen consumed by aerobic microbes in order to decompose this organic matter. As many microbes present in the water, the demand for oxygen will be high hence level of BOD will be high. Nitrates and phosphates acts as the plant nutrients, which leads aquatic life to grow, after their death they become organic waste in the water which then get decomposed by the aerobic microbes, hence resulting in a high BOD level.

With the increases in BOD level of water, dissolved oxygen level decreases, as oxygen is consumed by microbes. Since dissolved oxygen is less, aquatic life may not survive in that water (Riedel, Renneberg et al. 1988).

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Collection of sample and processing

Cyanide containing waste water was collected from coke-coal unit of Rourkela Steel Plant (BOD plant). Overview of the unit is as follows:

Gas from the effluent of coke-coal unit

↓

Transferred to water; purified gas liberated in the air

↓

Water with contaminants is transferred to equalization tank I and II for settlement of tar; settled tar is removed by sedimentation process

↓

Water is transferred to dissolved air floatation unit (DAF) and dosing pump house; removal of oil takes place by entrapment of oil in air bubbles

↓

Ferrous sulfate is used as a coagulant to separate suspended solids

↓

Aeration tank I (removal of phenol using *Pseudomonas* bacteria) + carry flocculator (removal of suspended particles)

↓

Trickled bed filter for removal of cyanide

↓

Aeration tank II (removal of ammonia using *nitrosomonas* bacteria) + carry flocculator (removal of suspended particles)

Sample was collected from outlet of DAF and dosing pump house. It was autoclaved at 121°C and 15 lb pressure for sterilization followed by centrifugation at 6000 rpm for 20 minutes for removal of contaminants and suspended particles.

3.2 Characterization of the cyanide containing waste water

Characterization was done by considering following points:

- pH
- Colour

- Odour
- Total dissolved solids
- Dissolved oxygen
- Biochemical oxygen demand

3.2.1 pH:

pH of the water was determined using digital pH meter

3.2.2 Colour:

Colour of the water was determined by visual analysis

3.2.3 Odour:

Odour of the water was determined by its nascent smell

3.2.4 Total dissolved solids:

An evaporating dish of suitable size was taken; it was cleaned, dried and weighed. 250-500 mL of the sample was filtered through a Whatman's filter paper no.4. Filtrate was taken in an evaporating dish. Sample was evaporated in a hot water bath. When whole water was evaporated, weight of evaporating dish was noted after cooling it in a desiccator.

Calculation:

$$TDS\ \left(\frac{g}{L}\right) = \frac{A - B}{V} * 100$$

Where, TDS = Total dissolved solids; A= final weight of evaporating dish (g); B= Initial weight of evaporating dish (g) and V= volume of sample taken (mL).

3.2.5 Dissolved oxygen

A glass stopper BOD bottle of known volume (250-300 mL) was taken and filled with sample avoiding bubbling. No air should be trapped in the bottle after stopper is placed. Bottle was opened and 2mL of each manganous sulphate and alkaline potassium iodide solutions were

added to the bottle using separate pipettes. After precipitate appeared, stopper was placed and bottle was shaken thoroughly. 2 ml of sulphuric acid was added to the bottle to dissolve precipitate. 50 ml content of the bottle was transferred in a conical flask. Few drops of starch indicator were added to the flask and titrated against sodium thiosulphate solution. End point was noted down where initial blue colour turned to colourless.

Reagent preparation:

A. **Sodium thiosulphate solution (0.025 N):** 6.205 g of sodium thiosulphate was dissolved in previously boiled distilled water and volume was made up to 1 litre. A pellet of sodium hydroxide was added to this as a preservative and stored in a coloured bottle.

B. **Manganous sulphate solution:** 100 g of manganous sulphate was dissolved in 200 ml previously boiled distilled water and solution was filtered.

C. **Alkaline potassium iodide solution:** 100 g of potassium hydroxide and 50 g of potassium iodide was dissolved in 200 ml previously boiled distilled water and solution was filtered.

D. **Starch indicator:** 1 g of starch was dissolved in 100 ml warm distilled water and few drops of toluene were added to this as a preservative.

Calculation:

$$\mathrm{DO}\left(\frac{\mathrm{mg}}{\mathrm{l}}\right) = \frac{\mathrm{V1} * \mathrm{N} * 8 * 1000}{\mathrm{V4}(\frac{\mathrm{V2}-\mathrm{V3}}{\mathrm{V2}})}$$

Where, DO = dissolved oxygen; V1 = volume of titrant (ml); N = normality of titrant (0.025); V2 = volume of sampling bottle after placing the stopper (ml); V3 = volume of manganous sulphate + potassium iodide solution added (ml); and V4 = volume of fraction of the contents used for titration (ml).

3.2.6 Biochemical oxygen demand

To prepare dilution water, BOD-free distilled water was aerated for about half an hour. Per litre of this water was added with 1 ml of each phosphate buffer solution, magnesium sulphate solution, calcium chloride solution and ferric chloride solution. pH of the sample was adjusted to neutrality. To ensure that not all oxygen of the sample is exhausted during incubation, sample was diluted with dilution water as per its expected BOD, (for this research study water was diluted with a dilution factor of 100). Two sets of BOD bottles were filled with this water sample. Total dissolved oxygen content (D_0) was determined in one set immediately using Winkler's method. Other set of BOD bottle was incubated at 20°C for five days in a BOD incubator. Bottle was taken out after five days and its dissolved oxygen content (D_5) was determined.

Calculation:

$$BOD_5 \text{ (mg/l)} = (D_0 - D_5) * \text{Dilution factor}$$

Where, D_0 = initial dissolved oxygen in the sample (mg/l) and D_5 = dissolved oxygen left out in the sample after 5 days incubation (mg/l).

3.3 Bacterial isolation and screening

Original cyanide containing waste water sample was taken in 1 ml amount in a cleaned glass test tube; added to 9 ml distilled water and shaken gently.1 ml of the suspension was taken and mixed with another 9 ml sterilized water followed by serial dilution up to 10^{-9} times. 10^{-9} to 10^{-7} times dilutions were plated over solid growth medium using spread plate technique and incubated for 48 hrs at 30ºC.Then grown colonies were picked up from the plates and again sub cultured in the plates containing growth medium using streak plate technique. The purification process was repeated twice and then colony characteristics of the purified strains were observed.

Media composition:

Nutrient media was used for the growth of microorganisms, composition is as given below(Atlas 2010):

Ingredients	Grams/litre
Peptic digest of animal tissue	5
Sodium chloride	5
Beef extract	1.5
Yeast extract	1.5
Agar	15
Final pH (at 25°C)	7.4±0.2

3.4 Gram's staining

A clean glass slide was taken washed and air dried followed by its sterilization with 70 percent ethanol. A thin bacterial colony was picked with an inoculation loop and thin smear was prepared on glass slide. Smear was air dried and heat fixed with the help of blue flame of spirit lamp. It was then stained with primary stain crystal violet; kept for one minute and was washed with distilled water. Then few drops of Gram's iodine were added to the smear and kept for one minute followed by washing with distilled water. Slide was then flooded and washed with absolute ethanol for 15 seconds. After this, smear was stained with the counter stain safranin; kept for one minute and washed with distilled water. Slide was then air dried and viewed using a compound microscope.

3.5 Biochemical characterization

Following biochemical tests were performed in order to characterize the bacterial flora:

- MR Test
- VP Test
- Catalase Test
- Oxidase Test
- Urease Test
- Starch hydrolysis test

3.5.1 MR test

Methyl Red-Voges- Proskauer (MR-VP) broth was used to grow microorganism and incubated for 48 hours at 30°C. After incubation, methyl red reagent was added to the broth in a small amount. If broth's color changes to red it shows positive result while appearance of yellow color shows negative result of the test.

Composition of MR-VP broth:

Ingredients	Grams/litre
Peptone	5
NaCl	5
Glucose	10
pH	7.2

3.5.2 VP test

After incubation of bacteria for 48 hours at 30°C in MR-VP broth, a mixed solution of α-naphthol and potassium hydroxide was added to the bacterial broth culture. Crimson red color formation shows positive result.

3.5.3 Catalase test

From a 24-48 hours incubated bacterial culture, a fully grown colony was picked and spread over a clean and dried slide. Then a drop of hydrogen peroxide was put over it. Presence or absence of bubbles was observed. Presence of bubbles shows a positive result, whereas absence shows a negative result.

3.5.4 Oxidase test

The presence of Oxidase enzyme is detected by this test. A pre inoculated bacterial broth culture was taken and few drops of methylene blue were added to the culture. If colour changes to colourless it shows positive result.

3.5.5 Urease test

Bacterial cultures were inoculated into the tubes containing urease broth and incubated at 30 °C for 72 hours. If organisms have urease enzyme it will split urea into ammonia and CO_2. This is detected by the change in the color of broth from yellow to purplish pink.

Composition for urease broth is as follows:

Ingredients	Grams/litre
Peptone	1
NaCl	5
K2HPO4	2
Glucose	1
Urea	20
pH	6.8
Phenol red	6 ml

3.5.6 Starch hydrolysis test

Nutrient agar plate containing 1% soluble starch was inoculated using inoculation loop in a circular manner, and incubated for 24 hours at 30°C. Iodine solution was added to this plate for 5 minutes and plate was observed for the formation of a clear zone around the colony.

Reagent preparation:

- **Iodine solution**

Iodine	1 gm
Potassium Iodide (KI)	2 gm
Distilled water	300ml

3.6 Analysis of cyanide degradation capacity of the isolated species

Preliminary experiments were performed for the selection of potential strain. To evaluate cyanide degradation of each bacterial strain, experiment was performed with all the isolated

bacterial strains individually. Experiments were carried out for 100 ml nutrient media in 250 ml conical flask for 60 hours at 30°C.

Sample was collected after equal intervals of 6 hours and analyzed for efficiency of bacteria to degrade cyanide.

The experiment was carried out by two different methods:

3.6.1 Method I

Media composition:

Ingredients	Grams/litre
Sodium chloride	0.5
Beef extract	0.15
Distilled water	100 ml
Cyanide containing RSP water	1 ml

3.6.2 Method II

Media composition:

Ingredients	Grams/litre
Sodium chloride	0.5
Beef extract	0.15
Cyanide containing RSP water	100 ml

3.7 To study the effect of pH on cyanide degradation capacity of bacterial strains

Two growth media were prepared as described in section 3.6, autoclaved and centrifuged for sterilization and removal of contaminants respectively. Bacterial strains were inoculated in the medium and were kept at three different pH values for 30°C. Finally cyanide degradation was

analyzed with the help of Expandable Ion Analyzer, model no. EA 940 (Thermo Orion)at equal intervals of 6 hours and study was carried out for 60 hours.

3.8 Parameters optimization influencing degradation of cyanide

Experiments for optimization of degradation of cyanide was carried out based on following parameters:

- pH
- Temperature
- Inoculum size

Media was prepared using beef extract as carbon source and sodium chloride to maintain osmotic equilibrium as described in section 3.6. Three levels of pH, temperature and inoculum size were varied during each experimental run. After each 6 hours sample was analyzed, the process was continued till 60 hours.

3.9 Preparation of the adsorbents

Granular activated carbon (GAC) particles were purchased from Himedia, India; in a size range of 1-2 cm. Clinker (a waste product from RSP) was collected in three different sizes. Both of the adsorbents were sterilized by autoclaving at 121°C, 15 lb pressure for 20 minutes (Dash, Balomajumder et al. 2008). Processed by soaking of the particles in 500 ml of 5 mol/L hydrochloric acid for 3 hours, followed by washing with distilled water thrice and drying in oven at 378.15 K(Li, Gong et al. 2012).

3.9.1 Study parameters for clinker

Clinker was studied on the basis of three different parameters as:

- pH
- Particle size
- Particle concentration

For pH study: acidic, basic and neutral pH was selected. For size, particles ranging from 0.5-1 cm, 1-2 cm and 2-3 cm were used whereas for concentration, particles of 1 gm, 2 gm and 3 gm were taken respectively and cyanide degradation was analyzed over a period of 60 hours.

3.9.2 Comparative study between GAC and clinker

A comparative study between GAC and clinker was carried out; in which particle size, particle concentration and pH was kept as 1-2 cm, 3 gm and neutral pH (7.2) respectively, which for both the adsorbents.

3.10 Simultaneous adsorption and biodegradation

For simultaneous adsorption and biodegradation, **clinker** was used with **Creamy (NAM 10^{-1}).** Nutrient media was prepared as given in section 3.6, set the pH to neutral and autoclaved. Adsorbent was prepared as mentioned in section 3.9. After autoclaving, media was inoculated with the bacterial strain and added with 3gm clinker. It was then kept in incubator at 30°C and analyzed after each 6 hours for 60 hours of study.

CHAPTER 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 Collection of sample

Sample was collected from outlet of DAF and dosing pump house as it was free of oil, grease, tar and microbial flora.

4.2 Physical characterization of cyanide containing waste water

Characterization was done on the water sample collected from aeration tank I and the result is shown in TABLE 1.

Table 1: Characterization of the water sample

Serial no.	Tests performed	Result
1	pH	8.28
2	Colour	Blackish brown
3	Odour	Pungent
4	Total dissolved solids (g/L)	9
5	Dissolved oxygen (mg/L)	6.48
6	Biochemical oxygen demand (mg/L)	256.61

4.3 Bacterial isolation and screening

For bacterial isolation, sample was collected from aeration tank I, where growth of microbial flora was observed after analysis. Four different colonies were selected after spread plate method and pure cultures were obtained after two subculture.

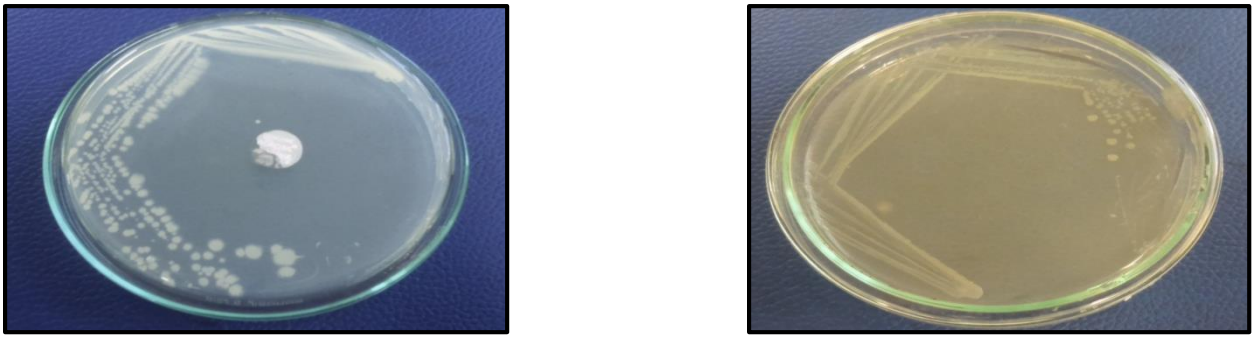


Fig.1. Streak plate method for bacterial colonies

Table 2: Colony characteristics of the isolated bacteria

Colony characteristics	**Spreaded (NAM 10^{-1})**	**Orange (NAM 10^{-1})**	**Creamy (NAM 10^{-1})**	**Dotted (KM 10^{-1})**
Size	0.15 cm	0.2 cm	0.15 cm	0.15 cm
Shape	Irregular	Wavy	Round	Round
Texture	Smooth	Smooth, shiny	Mucoid	Mucoid
Colour	Yellow	Orange	Pale yellow	Pale yellow
Margin	Irregular	Wavy	Entire	Entire
Elevation	Raised	Convex	Convex	Convex
Odour	Nil	Nil	Nil	Nil
Opacity	Transluscent	Transluscent	Transluscent	Transluscent

4.4 Selection of potential cyanide degrading strain

The percentage of cyanide degraded by each bacterial colony was analyzed by two methods that are described in section 3.6. Biodegradation efficiencies of the strains were observed for a period of 60 hours and results are tabulated in TABLE 3 and 4. It is evidence that maximum degradation of cyanide was removed by bacterial colony **Creamy (NAM 10^{-1})**using method I; whereas bacterial colony **Spreaded (NAM 10^{-1})**shows maximum cyanide degradation using method II**.** Hence, these two strains were selected for further cyanide degradation studies.

4.4.1 Method I:Creamy (NAM 10^{-1})

Table 3: Percentage of cyanide degraded by bacterial strains by method I

Sr no.	hours	Spreaded (NAM 10^{-1})	Orange (NAM 10^{-1})	Creamy (NAM 10^{-1})	Dotted (KM 10^{-1})
1	0	10.3	10.3	10.3	10.3
2	12	4.49	4.45	4.43	4.42
3	24	3.20	3.37	3.42	2.97
4	36	2.48	2.58	2.12	1.71
5	48	1.97	1.96	1.67	1.53
6	60	1.48	1.51	1.39	1.42
Percent degradation	**60**	**85.63**	**85.33**	**86.50**	**86.21**

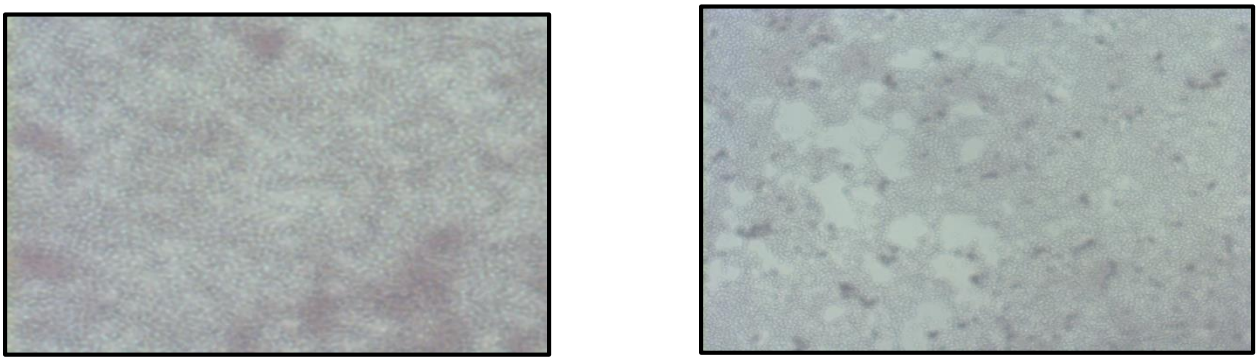
4.4.2 Method II: Spreaded (NAM 10^{-1})

Table 4: Percentage of cyanide degraded by bacterial strains by method II

Sr no.	hours	Spreaded (NAM 10^{-1})	Orange (NAM 10^{-1})	Creamy (NAM 10^{-1})	Dotted (KM 10^{-1})
1	0	10.3	10.3	10.3	10.3
2	12	3.60	3.66	3.62	3.68
3	24	2.69	2.82	2.76	2.89
4	36	1.87	2.02	1.96	2.17
5	48	1.59	1.79	1.76	1.82
6	60	1.42	1.51	1.49	1.53
Percent degradation	**60**	**86.21**	**85.33**	**85.53**	**85.14**

4.5 Gram's staining images of Creamy (NAM 10^{-1}) and Spreaded (NAM 10^{-1})

Gram's staining was done in order to find out the morphological characteristics of the bacterial cultures. Results obtained as shown in Fig. 2 and discussed below.



Creamy (NAM 10^{-1}) **Spreaded (NAM 10^{-1})**

Fig.2. Gram's staining images of bacterial colonies

From the above images, it can be concluded that Creamy (NAM 10^{-1}) is a Gram negative cocci shaped bacteria; whereas Spreaded (NAM 10^{-1}) is a Gram positive cocci shaped bacteria.

4.6 Biochemical Characterization Of The Efficient Strains

4.6.1 MR test

This test is performed to detect ability of microorganism to produce acid using glucose. Because of production of the acid, pH of the medium lowers below 4.2, which can then be detected by methyl red, a pH indicator. If red colour appears after addition of the indicator, the test is considered to be positive; whereas if colour does not change, the test is considered to be negative.

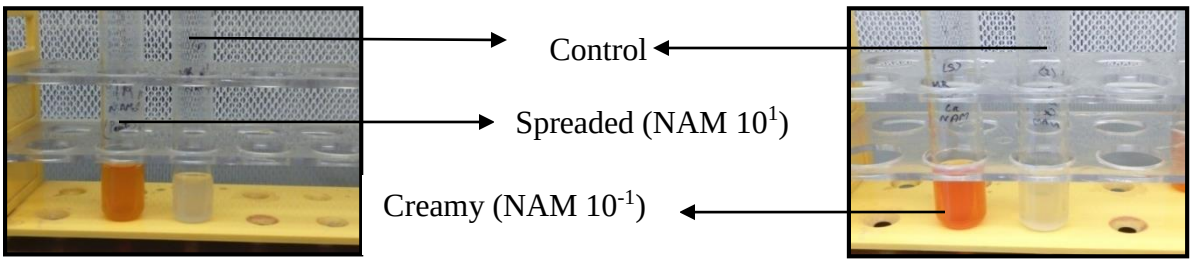


Fig 3: MR test for bacteria Spreaded (NAM 10^{-1}) and Creamy (NAM 10^{-1})

Both the bacterial cultures show positive result for MR test. Hence, it can be concluded that both bacteria can produce acid using glucose.

4.6.2 VP test

This test is used to check the ability of bacteria to ferment carbohydrates with the production of acetyl methyl carbinol reduction into neutral products and CO_2. Development of crimson colour shows a positive result; whereas no colour change indicates a negative result.

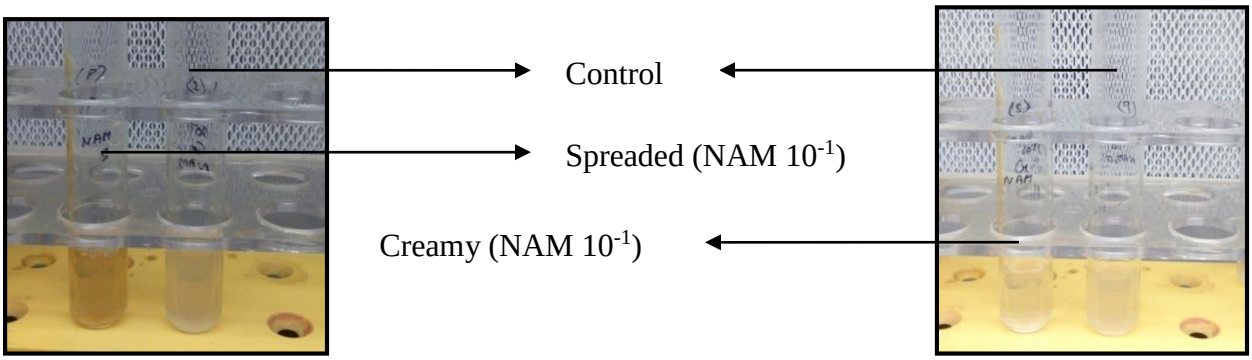


Fig 4: VP test for bacteria Spreaded (NAM 10^{-1}) and Creamy (NAM 10^{-1})

Both the bacterial cultures show negative result for VP test. Hence, it can be concluded that both bacteria cannot ferment carbohydrates.

4.6.3 Catalase test

Catalase enzyme produced by the microorganisms, splits hydrogen peroxide (H_2O_2) into water and oxygen, which leads to formation of bubbles. Presence of bubbles indicates positive result; whereas no bubble formation indicates negative result.

Creamy (NAM 10^{-1})

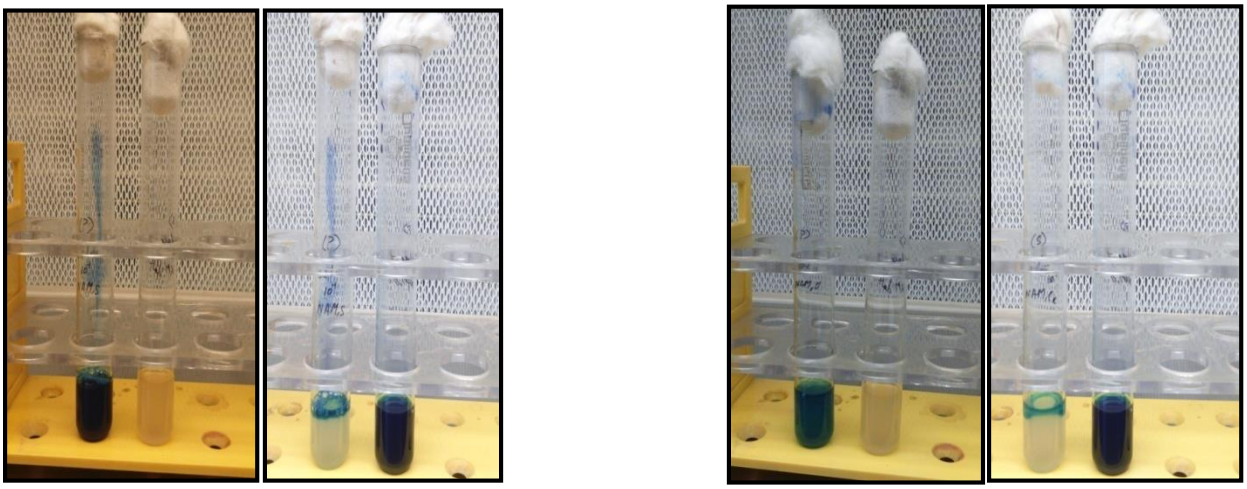
Spreaded (NAM 10^{-1})

Fig 5: Catalase test for bacteria Spreaded (NAM 10^{-1}) and Creamy (NAM 10^{-1})

From the above figures it can be seen that there is no formation of bubbles; hence it can be concluded that both the bacterial cultures does not produce enzyme Catalase.

4.6.4 Oxidase test

The enzyme Oxidase produced by microorganism, catalyses transport of electrons between bacteria and redox dye (methylene blue). Because of this transport, the dark blue colour of the dye turns colourless indicating positive result; whereas persistence of colour indicates negative result.



Before After Before After

Spreaded (NAM 10^{-1}) **Creamy (NAM 10^{-1})**

Fig 6: Oxidase test for bacteria Spreaded (NAM 10^{-1}) and Creamy (NAM 10^{-1})

It can be concluded from the results that, both the bacterial cultures produce enzyme Oxidase hence, showing positive result.

4.6.5 Urease test

Presence of Urease enzyme is detected by this test. Urease enzyme produced by microorganisms splits urea into ammonia and carbon dioxide leading to the formation of purplish pink colour, indicating positive result; whereas no change in colour indicates negative result.

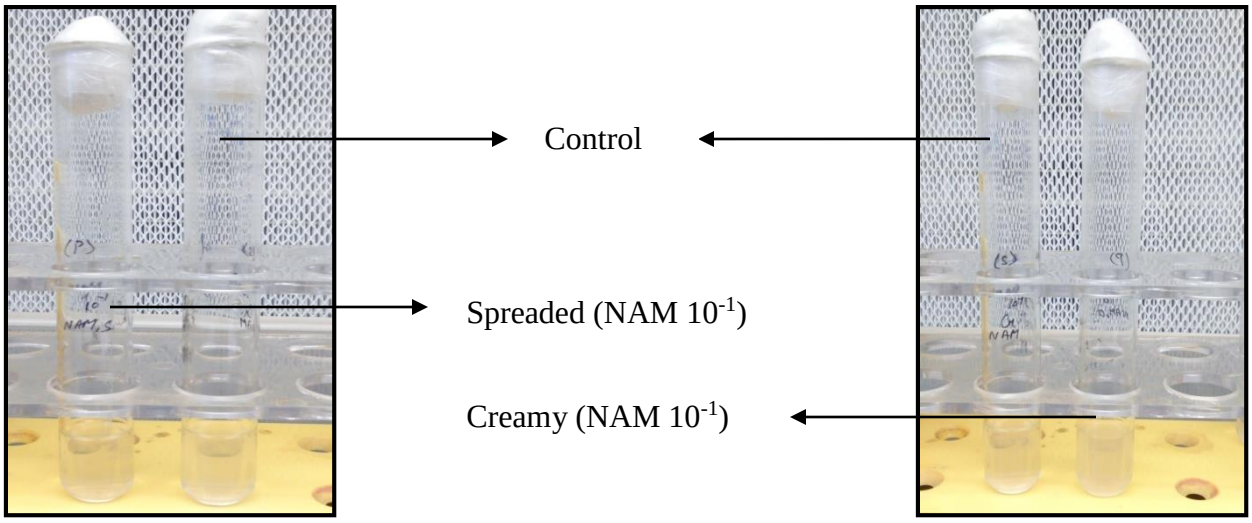
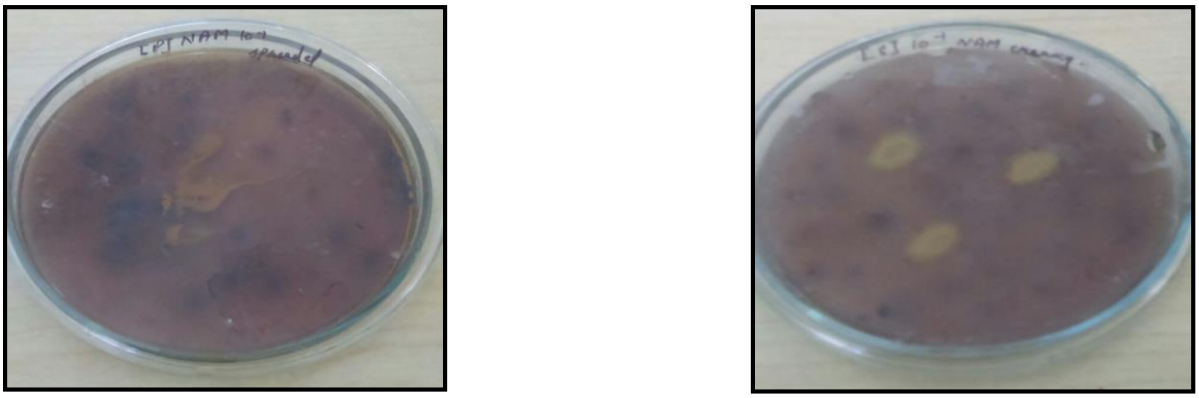


Fig 7: Urease test for bacteria Spreaded (NAM 10^{-1}) and Creamy (NAM 10^{-1})

4.6.6 Starch hydrolysis test

Presence of Amylase enzyme in the microbe, which hydrolyses starch into simple substances like glucose, dextrin etc. is detected by this test. Development of a clear zone around the colony indicates positive result.



Spreaded (NAM 10^{-1}) **Creamy (NAM 10^{-1})**

Fig 8: Starch hydrolysis test for bacteria Spreaded (NAM 10^{-1}) and Creamy (NAM 10^{-1})

From the above figures, it can be concluded that no clear zone was observed around both the bacterial cultures indicating a negative result for the presence of enzyme Amylase.

4.7 Effect of different pH on degradation capacity of the bacterial strains

The following bar graph shows behavior of the bacterial strains on the degradation efficiency of cyanide at different pH values. It can be seen that for both the bacterial cultures, maximum degradation was obtained at neutral pH values that is 6.5-7; followed by basic pH that is 8.5-9 and least was observed for acidic pH that is 3.5-4.

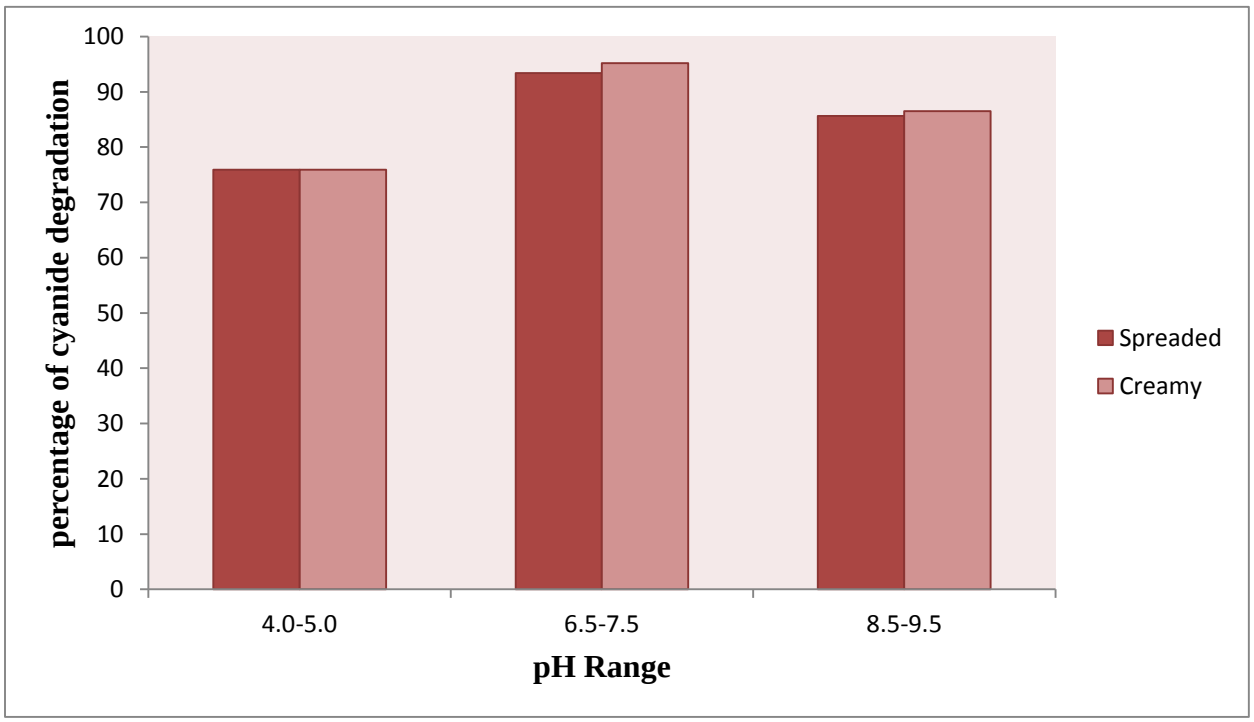


Fig9: Effect of different pH on cyanide degradation capacity of bacterial strains

4.8 Optimization of pH, Temperature and inoculum size

Biodegradation of cyanide is highly influenced by pH, temperature and inoculum size. Both pH and temperature is essential for growth of the cyanide degrading bacteria. Moreover, pH is considered to play a vital role in cyanide degradation. With the above mentioned parameters, inoculum size as well as oxygen supply is also necessary for the cyanide degradation efficiency. Initial dissolved concentration of the sample water was determined using Winkler's method.

Cyanide degradation experiment was carried out by standard bacterial degradation method. The experiments were performed in triplicates and observations are tabulated in TABLE 5 and 6.

TABLE 5:Effect of temperature, pH and inoculum size on cyanide degradation by Spreaded (NAM 10^{-1})

Sr no.	Temperature (°C)	pH	Inoculum size (ml)	Percent degradation (%)
1	20	3.5-4.5	0.5	68.24
			1	85.56
			1.5	90.04
2		6.5-7.5	0.5	81.12
			1	87.57
			1.5	90.68
3		8.5-9.5	0.5	72.68
			1	84.56
			1.5	89.97
4	30	3.5-4.5	0.5	71.21
			1	92.87
			1.5	93.96
5		6.5-7.5	0.5	74.27
			1	94.54
			1.5	94.74
6		8.5-9.5	0.5	72.77
			1	93.54
			1.5	94.21
7	40	3.5-4.5	0.5	68.54
			1	86.20
			1.5	90.52
8		6.5-7.5	0.5	87.62
			1	90.55
			1.5	91.71
9		8.5-9.5	0.5	77.58
			1	85.84
			1.5	89.92

Above table gives a description of optimization studies on parameters like pH, temperature and concentration. It can be seen that, Spreaded (NAM 10^{-1}) shows highest cyanide efficiency of **94.74%**in neutral pH that is 6.5-7.5 at 30°C in a concentration of 1.5 mL.

TABLE 6:Effect of temperature, pH and inoculum size on cyanide degradation by Creamy (NAM 10^{-1})

Sr no.	Temperature (°C)	pH	Inoculum size (ml)	Percent degradation (%)
1	20	3.5-4.5	0.5	63.04
			1	90.34
			1.5	89.42
2		6.5-7.5	0.5	78.05
			1	92.34
			1.5	92.30
3		8.5-9.5	0.5	79.65
			1	91.93
			1.5	91.80
4	30	3.5-4.5	0.5	68.42
			1	92.03
			1.5	91.02
5		6.5-7.5	0.5	80.87
			1	96.65
			1.5	95.05
6		8.5-9.5	0.5	80.15
			1	96.02
			1.5	95.00
7	40	3.5-4.5	0.5	62.48
			1	90.33
			1.5	88.42
8		6.5-7.5	0.5	80.10
			1	93.22
			1.5	92.51
9		8.5-9.5	0.5	78.82
			1	91.82
			1.5	89.23

Above table gives a description of optimization studies on parameters like pH, temperature and concentration. It can be seen that, Creamy (NAM 10^{-1}) shows highest cyanide efficiency of **96.65%** in neutral pH that is 6.5-7.5 at 30°C in a concentration of 1 mL.

4.9 Adsorbents: Granular Activated Carbon (GAC) and clinker

SEM (Scanning Electron Microscopy) images were taken at different magnifications in the range of 50X to 100X in order to find out the difference in pore diameter.

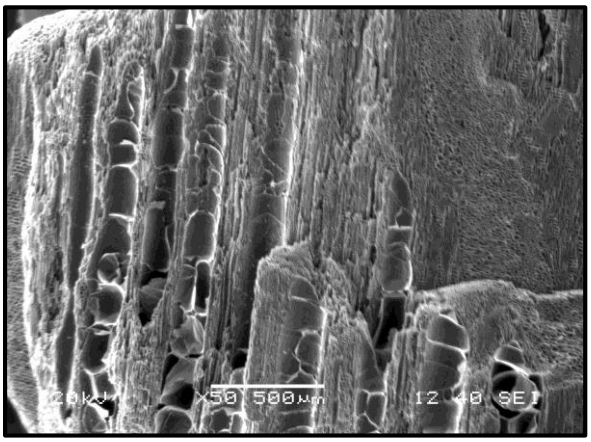


Fig. 10: Granular Activated Carbon at 50 X magnification

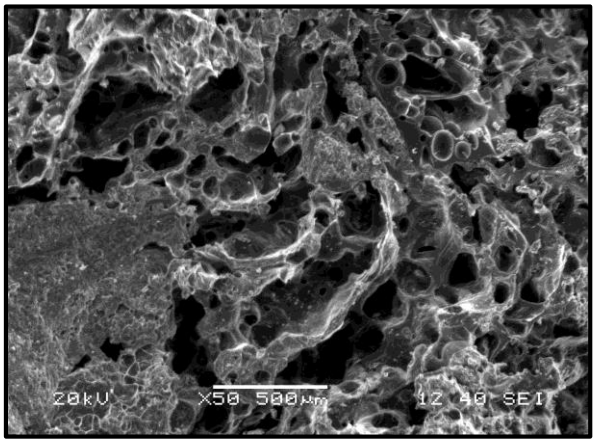


Fig. 11: Clinkerat 50 X magnification

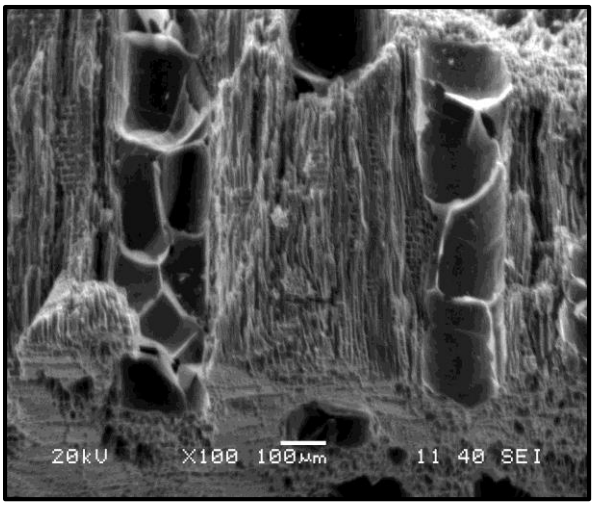


Fig. 12: Granular Activated Carbon at 100 X magnification

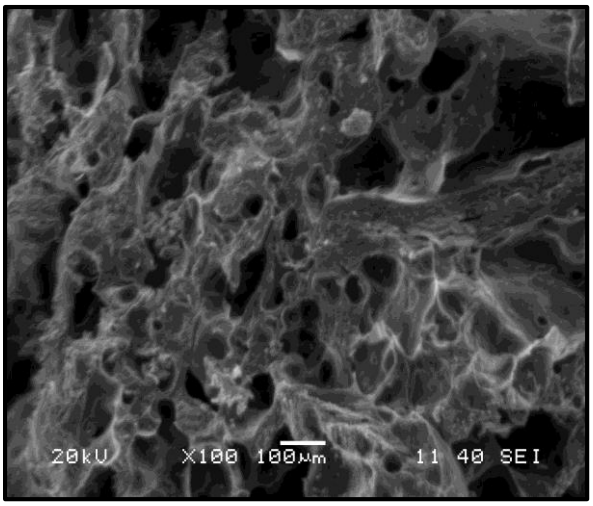


Fig. 13 Clinkerat 100 X magnification

From the above figures it can be seen that, GAC has smaller pore size as compared to Clinker because of which it shows good adsorption efficiency.

4.10 Clinker as an adsorbent

Clinker is a waste product of coal, generated from RSP. Following parameters were analyzed in order to check the adsorption capacity of clinker.

4.10.1 Effect of pH

TABLE 7: Effect of pH on cyanide removal capacity of clinker

Sr no.	Time	Acidic (4-5)	Neutral (6.5-7.5)	Basic (8.5-9.5)
1	0	10.3	10.3	10.3
2	24	4.89	5.01	5.56
3	48	3.02	3.20	3.58
4	72	1.59	1.73	1.92
Percent adsorption (%)	**72**	**84.56**	**83.20**	**81.35**

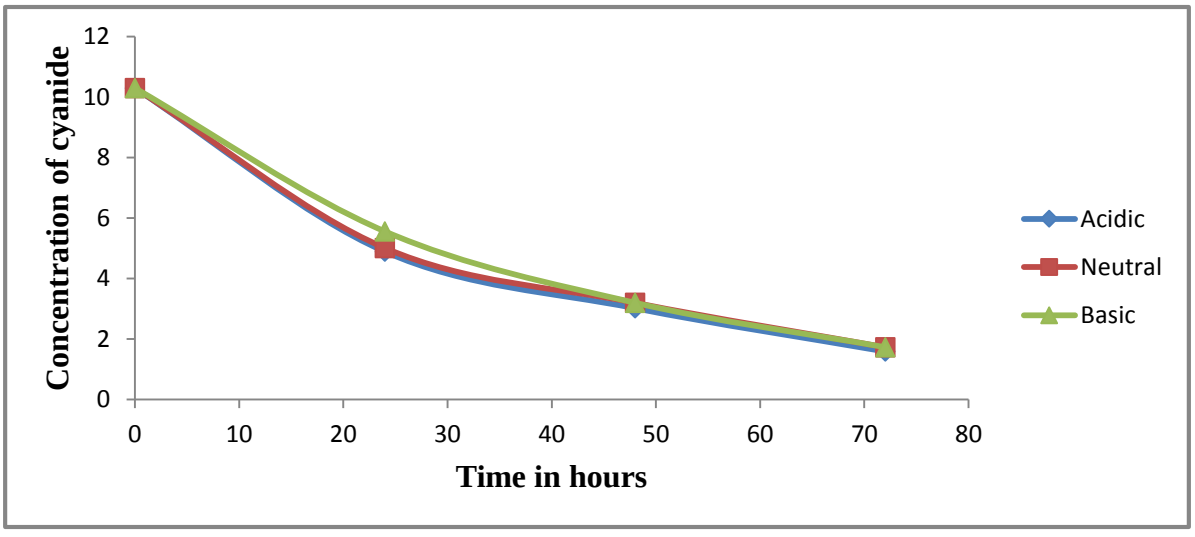


Fig 14: Effect of pH on cyanide removal capacity of clinker

From the above figure it can be seen that clinker shows maximum cyanide removal of **84.56%** after 72 hours of analysis at acidic pH that is the pH range of 4-5, followed by neutral and basic pH.

4.10.2 Effect of size

TABLE 8: Effect of size on cyanide removal capacity of clinker

Sr no.	Time	Powdered form of clinker	2-6 mm	6-10 mm
1	0	10.3	10.3	10.3
2	24	7.28	6.53	7.58
3	48	5.69	5.38	5.92
4	72	2.96	2.20	2.48
Percent adsorption (%)	**72**	**71.26**	**78.64**	**75.92**

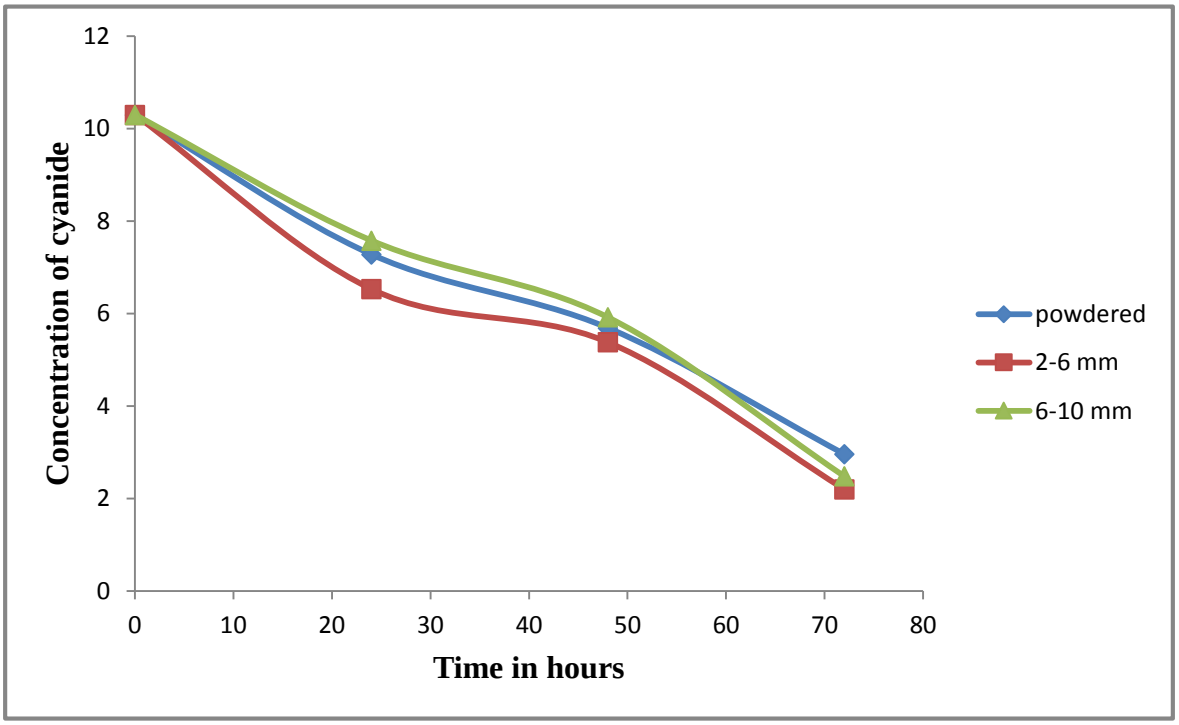


Fig 15: Effect of size on cyanide removal capacity of clinker

From the above figure it can be seen that clinker shows maximum cyanide removal of **78.64%** after 72 hours of analysis at a size of 2-6 mm, followed by size range of 6-10 mm and powdered.

4.10.3 Effect of concentration

TABLE 9: Effect of concentration on cyanide removal capacity of clinker

Sr no.	Time	1 gm	2 gm	3 gm
1	0	10.3	10.3	10.3
2	24	5.93	5.63	7.28
3	48	3.49	3.21	2.93
4	72	2.13	1.764	1.650
Percent adsorption (%)	**72**	**79.32**	**82.87**	**83.98**

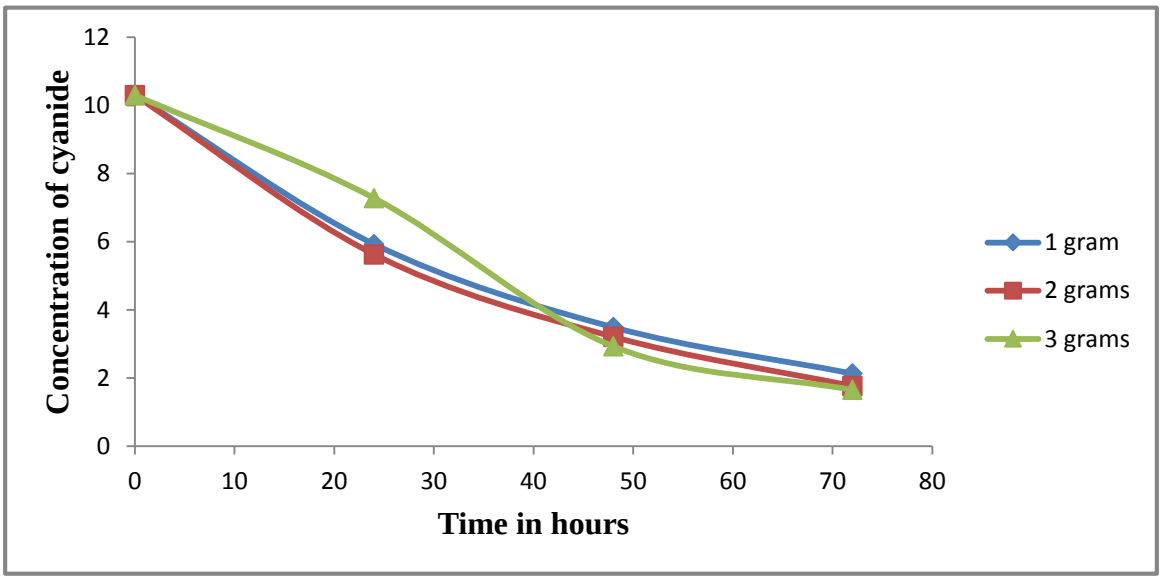


Fig 16: Effect of concentration on cyanide removal capacity of clinker

From the above figure it can be seen that clinker shows maximum cyanide removal of **83.98%** after 72 hours of analysis at a concentration of 3 gm, followed by concentration of 2 gm and 1 gm. It can be concluded that, clinker shows maximum adsorption at **acidic pH, with a size of 2-6 mm and in a concentration of 3 gm/ 25 mL.**

4.11 Comparative study between Granular Activated Carbon (GAC) and clinker

3 gm of each adsorbent was added to 25 mL of cyanide containing RSP waste water and analyzed for cyanide removal.

TABLE 10: Comparative study between Granular Activated Carbon (GAC) and clinker

Sr no.	Time	Clinker	GAC
1	0	10.3	10.3
2	24	6.93	6.28
3	48	4.49	3.93
4	72	1.13	0.650
Percent adsorption (%)	**72**	**89.02**	**93.68**

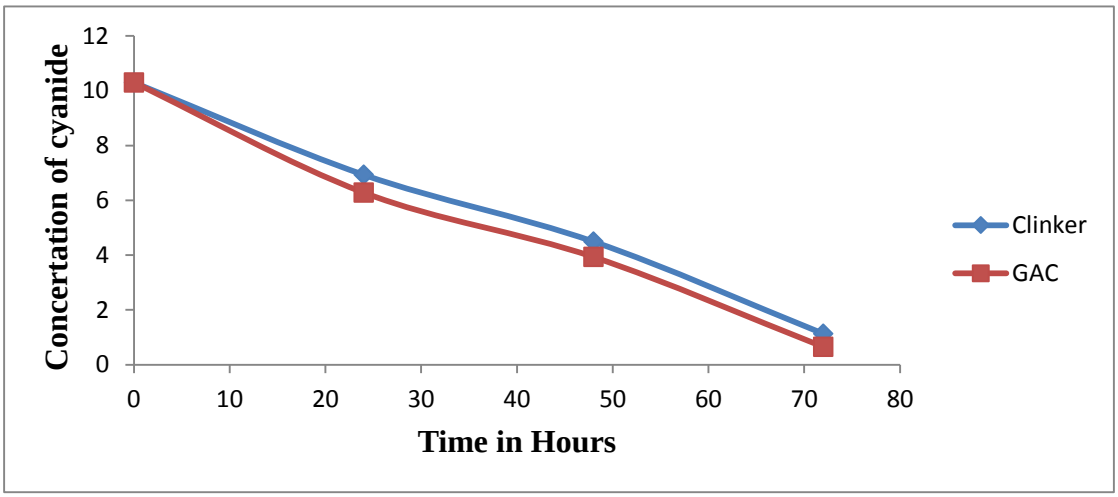


Fig 17: Comparative study between Granular Activated Carbon (GAC) and clinker

It can be seen that, GAC shows more cyanide removal over a period of 72 hours as compared to clinker; but difference is not much and hence clinker can also be used as an adsorbent for cyanide removal.

4.12 Simultaneous adsorption and biodegradation

For SAB process bacterial culture **Creamy (NAM 10^{-1})** along with **clinker** as an adsorbent was used and analyzed for cyanide degradation over a period of 60 hours.

Sr no.	Hours	Creamy (NAM 10^{-1})+ Clinker
1	0	10.3
2	12	2.73
3	24	2.53
4	36	1.87
5	48	0.912
6	60	0.086
Percent degradation (%)	**60**	**99.16**

TABLE 11: Simultaneous adsorption and biodegradation

From the above analysis, it can be concluded that using SAB process maximum cyanide degradation can be achieved. It may be due to microbes getting maximum surface area for immobilization; hence can stay on the adsorbent and degrade cyanide in a large amount.

CHAPTER 5

CONCLUSION

5. CONCLUSION

The present study deals with the degradation of cyanide from RSP waste water using different methods; including physical and biological methods. Bacterial colonies were successfully isolated from water sample in AT I and efficient cyanide degrading colony was screened. Various process parameters such as pH, temperature, inoculum size were studied and optimized for obtaining maximum cyanide degradation efficiency. Along with the biological method, adsorbents were also used for removal of cyanide. A comparative study between Granular Activated Carbon (GAC) and clinker was carried out in order to identify their adsorption capacities. Optimum conditions for biodegradation were obtained and a simultaneous adsorption and biodegradation (SAB) study was carried out at neutral pH of 6.5-7.5, temperature 30°C, and inoculum size of 1 mL. Using SAB process, the maximum cyanide degradation was found to be 99.16 % which is quite higher than the degradation achieved using other methods. Therefore, it has been demonstrated that SAB is the best method for achieving highest cyanide removal efficiency.

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